Immunity, Vol. 7, 25–36, July, 1997, Copyright ©1997 by Cell Press

EBF and E47 Collaborate to Induce Expression of the Endogenous Immunoglobulin Surrogate Light Chain Genes

Mikael Sigvardsson, Mary O'Riordan, and Rudolf Grosschedl
Howard Hughes Medical Institute
Departments of Microbiology and Biochemistry
University of California
San Francisco, California 94143–0414

Summary

Early B cell factor (EBF) and E47 participate in the transcriptional control of early B lymphocyte differentiation. With the aim of identifying genetic targets for these transcription factors, we stably transfected cDNAs encoding EBF or a covalent homodimer of E47, individually or together, into immature hematopoietic Ba/F3 cells, which lack both factors. In combination, EBF and E47 induce efficient expression of the endogenous immunoglobulin surrogate light chain genes, λ*5* and *VpreB*, whereas other pre–B cell–specific genes remain silent. Multiple functionally important EBF and E47 binding sites were identified in the λ*5* promoter/enhancer region, indicating that λ*5* is a direct genetic target for these transcription factors. Taken together, these data suggest that EBF and E47 synergize to activate expression of a subset of genes that define an early stage of the B cell lineage.

Introduction

B cell differentiation is a complex developmental process that ultimately generates antibody-secreting cells. This pathway involves multiple stages of differentiation that are defined by the expression of specific cell surface markers and the genomic rearrangement status of the immunoglobulin (Ig) loci. The earliest characterized committed B cell precursor expresses the cell surface markers B220 and AA4.1 and has the Ig heavy chain locus in its germline configuration (Hardy et al., 1991; Li et al., 1996). Subsequent differentiation generates pro–B cells that express the proteins λ5, VpreB, Igα, and Igβ and that rearrange the D and J segments of the Ig heavy chain locus (reviewed by Alt et al., 1987; Melchers et al., 1993). The proteins λ5 and VpreB serve as surrogate light chains of the pre–B cell receptor, which forms after completion of the V-to-DJ rearrangement of the Igμ heavy chain locus in pre–B cells (Karasuyama et al., 1990; Tsubata and Reth, 1990). In mature B cells, the surrogate light chains are replaced with functionally rearranged Igκ or Igλ light chains, generating an IgM antigen receptor (reviewed by Melchers et al., 1993; Borst et al., 1996). The Igα and Igβ proteins, encoded by the *mb1* and *B29* genes (Hombach et al., 1990a, 1990b) respectively, participate in signal transduction through both the pre–B cell receptor and the IgM receptor (Nakamura et al., 1992; Brouns et al., 1993; Papavasiliou et al., 1995). The functional importance of the pre–B and B cell receptors for cellular differentiation has been demonstrated by targeted inactivation of the genes encoding the various components of these receptors. Mice carrying mutations in the genes encoding the Igμ heavy chain (Ehlich et al., 1993), λ5 (Kitamura et al., 1992), or Igβ (Gong and Nussenzweig, 1996) result in an arrest of B cell differentiation at the pre–B cell stage.

Transcriptional control of B cell differentiation is critically dependent on three genes encoding the transcription factors EBF (early B cell factor) (Hagman et al., 1991, 1993), E47 (Murre et al., 1989a), and B cell–specific activitor protein (BSAP) (Barberis et al., 1990). Targeted inactivation of the genes encoding E47 or EBF results in a similar block of B cell differentiation, prior to the rearrangement of the Ig heavy chain locus (Bain et al., 1994; Zhuang et al., 1994; Lin and Grosschedl, 1995). However, both EBF- and E47-deficient mice contain some early B cell precursors that express B220 and the IL-7 receptor, raising the possibility that neither EBF nor E47 determines the cell fate of the B lymphocyte lineage (Bain et al., 1994; Lin and Grosschedl, 1995). Targeted inactivation of the *Pax5* gene encoding BSAP results in a block of B cell differentiation at a later stage, with expression of B cell markers like λ5 and VpreB and initiation of D-to-J recombination in the Ig heavy chain locus (Urbanek et al., 1994; Nutt et al. 1997).

EBF is a homodimeric transcription factor that is expressed in pro–B, pre–B, and B cells, but not in terminally differentiated plasma cells. In addition, EBF is expressed in adipose tissue and in olfactory neurons (Hagman et al., 1993; Wang and Reed, 1993). This transcription factor has been shown to interact with a functionally important site in the *mb1* promoter (Hagman et al., 1991) and some Ig promoters (Sigvardsson et al., 1996). EBF contains a novel DNA-binding motif that recognizes variants of the palindromic nucleotide sequence 5′-ATTCCCNNGGGAAT (Travis et al., 1993). Homodimerization of EBF is mediated by a structural motif that appears to be related to the dimerization motif of helix-loop-helix (HLH) proteins (Murre et al., 1989b; Hagman et al., 1993).

The *E2A* gene encodes two distinct proteins, E47 and E12, which are generated by alternative splicing, and are members of the basic helix-loop-helix (bHLH) family of transcription factors (Murre et al., 1989a, 1989b; reviewed by Kadesch, 1992; Murre et al., 1994). E2A proteins bind a consensus E-box sequence 5′-CANNTG, which is found in the transcriptional control regions of B cell–specific and ubiquitously expressed genes (Ephrussi et al., 1985; Murre et al., 1989b; Brennan and Olson, 1990). Both E47 and E12 recognize these sequences as heterodimers with other members of the family of bHLH proteins that are often restricted to specific cell types (Murre et al 1989b; Brennan and Olson 1990; Lassar et al., 1991; Hu et al., 1992). No B cell–specific bHLH partners for E47 and E12 have yet been identified (Shen and Kadesch, 1995). Instead, the B cell–specific function of E47 appears to be related to the formation of homodimers that are detected uniquely in B cells (Murre et al., 1991; Bain et al., 1993; Jacobs et al., 1994; Shen and Kadesch, 1995). The formation of

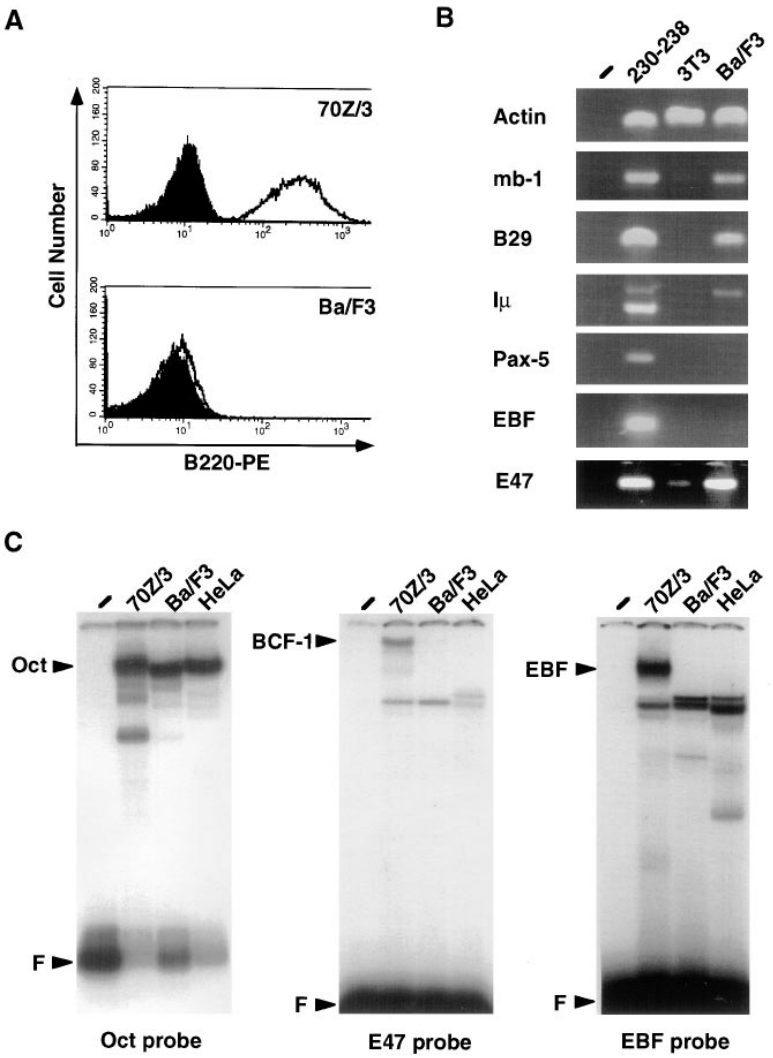

Figure 1. Ba/F3 Cells Display a Subset of B Cell Markers

(A) Ba/F3 cells do not express the surface antigen B220. Flow cytometry analysis of expression of the B cell lineage marker B220 on the surface of the pre–B cell line, 70Z/3, and on cells of the immature hematopoietic cell line Ba/F3. Filled areas represent the background phycoerythrin (PE) signal without antibody; open areas represent the phycoerythrin signal obtained after antibody addition.

(B) Ba/F3 cells express *Im*, *B29*, and *mb1* transcripts but not other early B cell markers. Ethidium bromide–stained agarose gels showing the products of a qualitative RT-PCR analysis from 30 cycles of total RNA from Ba/F3 cells, 230–238 pre–B cells, and NIH 3T3 fibroblasts.

(C) Ba/F3 cells lack the B cell–specific DNA-binding proteins, EBF and BCF-1. Electrophoretic mobility shift assays of nuclear extracts (5 μg) from Ba/F3 cells, 70Z/3 pre–B cells, or HeLa cells. The nuclear extracts were incubated with either ^{32}P-labeled octamer (Oct probe), *μE5* (E47 probe), or *mb1* (EBF probe) oligonucleotides. Arrowheads indicate the positions of the Oct–DNA, EBF–DNA, and BCF-1 (E47 homodimer) DNA complexes; F indicates migration of free probes.

an E47 homodimer, identified as the complex BCF-1, may involve the formation of a covalent disulfide bond (Benezra, 1995) and is possibly regulated by a B cell–specific dephosphorylation of the protein (Sloan et al., 1996).

A specific role for E47 in B cell differentiation was inferred not only from targeted gene inactivation but also from experiments examining the potential of E47 to induce a B cell–like phenotype in non–B cells. Ectopic expression of an E47 cDNA in non–B cells resulted in up-regulation of transcription from the genes encoding TdT, RAG-1, and Oct-2 and from the unrearranged μ locus (Schlissel et al., 1991; Choi et al., 1996). None of these putative target genes of E47 can account for the early block in B cell differentiation in mice lacking E47. Mice carrying targeted mutations in the genes encoding TdT (Gilfillan et al., 1993; Komori et al., 1993), RAG-1 (Mombaerts et al., 1992), RAG-2 (Shinkai et al., 1992), Oct-2 (Corcoran et al., 1993), and Igμ (Ehlich et al., 1993) generate pro–B cells that express the surrogate light chain λ5. In contast, mice deficient for E47 have an earlier block of B differentiation, prior to the expression of λ5 (Bain et al., 1994; Zhuang et al., 1994).

Although the targeted inactivations of *EBF* and *E2A* genes demonstrate an essential role of these transcription factors in early B cell differentiation, the absence of pro–B cells in these mice obscures the identification of critical genetic targets. Therefore, we examined whether EBF and E47 homodimers, singly or together, could activate B cell–specific genes that might account for the observed phenotype in the deficient mice. Here we show that the stable expression of EBF in the immature hematopoietic cell line Ba/F3 induces the expression of the surrogate light chain genes *λ5* and *VpreB* but not other lymphoid-specific genes such as *Rag1*, *IL7R*, *TdT*, and *Pax5*. In addition, we find that the expression of these genes is markedly increased by coexpression of forced dimers of E47, suggesting that these transcription factors collaborate in the activation of a subset of B cell–specific genes.

Results

Stable Ectopic Expression of EBF in Ba/F3 Cells Induces the Expression of λ5 and VpreB

To study the potential role of EBF and E47 in the regulation of B cell differentiation, we chose to express these

transcription factors in a nontransformed, immature hematopoietic cell line. Ba/F3 is a bone marrow–derived, IL-3–dependent cell line that may be committed to the B cell differentiation pathway (Palacios and Steinmetz, 1985). However, Ba/F3 cells have been shown to lack the B cell–specific homodimer of E47, termed BCF-1 (Murre et al., 1991; Shen and Kadesch, 1995), suggesting that these cells may represent either an early progenitor of B cells or a nonlymphoid cell type. To define Ba/F3 cells in relation to the B cell lineage more clearly, we analyzed the expression of various B cell markers (Figure 1). Flow cytometry with antibodies directed against B220 indicated that the Ba/F3 cells lack this pan–B cell marker, which was detected on the surface of 70Z/3 pre–B cells (Figure 1A). In addition, we examined Ba/F3 cells for the presence of B cell–specific transcripts by reverse transcriptase–directed polymerase chain reaction (RT-PCR). Ba/F3 cells were found to express mb-1, B29, germline $I\mu$ transcripts, and E47 (Figure 1B). These cells do not contain detectable transcripts from the *Pax5*, *EBF*, *TdT*, *Rag1*, *VpreB*, $\lambda 5$, CD19, or *IL7R* genes, which are expressed in the pre–B cell line 230–238 (Figure 1B, and data not shown). As expected, none of the B cell–specific genes examined are expressed in NIH 3T3 cells in which E47 transcripts were detected, although at a lower level. We also confirmed the absence of E47 homodimers (BCF-1) and EBF in Ba/F3 cells by analyzing nuclear extracts for the presence of proteins that recognize the E47 and EBF binding sites in an electrophoretic mobility shift assay (Figure 1C). Although *Oct*-binding proteins could be detected in Ba/F3 and 70Z/3 nuclear extracts at similar levels, BCF-1 and EBF were detected only in 70Z/3 nuclear extracts. This phenotype of Ba/F3 cells appeared to be clonally stable, and no additional B cell markers were found to be induced by culturing the Ba/F3 in the presence of IL-3 on stromal cells (data not shown). Thus, the Ba/F3 cell line may represent an early B cell precursor that lacks EBF and E47 homodimers, providing a good model system for functional studies of the role of these transcription factors in early B cell differentiation.

To examine the ability of EBF to induce the expression of B cell–specific genes, we stably transfected Ba/F3 cells with a construct encoding a T7-tagged EBF cDNA, together with the neomycin-resistance gene. We analyzed 25 neomycin-resistant clones for the expression of EBF by electrophoretic mobility assays. Five clones were identified that expressed EBF at levels ranging between 3% and 36% of the levels of endogenous EBF in 70Z/3 (Figure 2A). Analysis of these EBF-positive Ba/F3 clones for the expression of various B cell markers by RT-PCR analysis indicated that all five clones contained low levels of transcripts from the *VpreB* and $\lambda 5$ genes but not from the *IL7R*, *Pax5*, and *Rag1* genes (Figure 2B and data not shown). Neither *VpreB* nor $\lambda 5$ was expressed in the parental Ba/F3 cells or in any of 10 neomycin-resistant EBF-negative clones (data not shown). Thus, EBF can activate the expression of at least two B cell markers from a silent state.

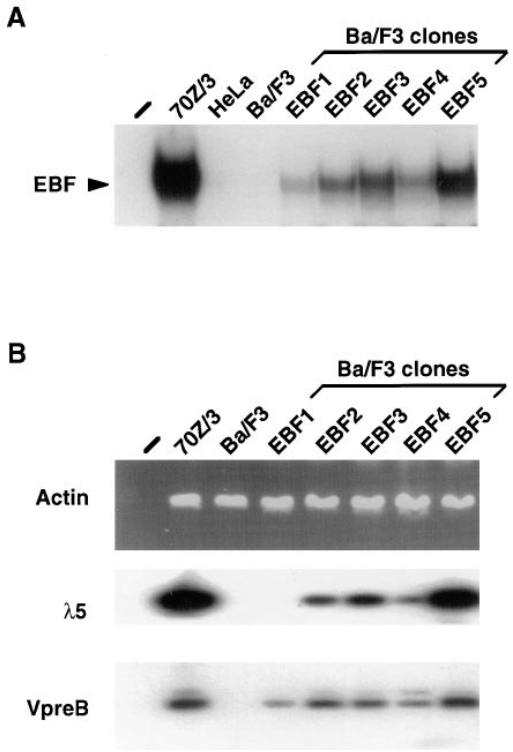

Figure 2. Expression of EBF in Ba/F3 Cells Induces Transcriptional Activation of the $\lambda 5$ and *VpreB* Genes at Low Levels

(A) Electrophoretic mobility shift assay to detect EBF expression in Ba/F3 cells stably transfected with an EBF cDNA. Nuclear extract (5 μg) from each cell clone was incubated with an EBF-binding site from the *mb1* promoter. Nuclear extracts from 70Z/3 pre–B cells and HeLa cells were used as positive and negative controls, respectively. The protein–DNA complexes were separated on a 6% polyacrylamide gel. The region of the EBF–DNA complex in the autoradiogram is shown, and the relative amounts of the complex were quantitated by PhosphorImager analysis.

(B) $\lambda 5$ and *VpreB* expression in EBF-positive Ba/F3 cells. Qualitative RT-PCR analysis of total RNA from parental and transfected Ba/F3 clones, and from 70Z/3 pre–B cells. (Top) An ethidium bromide–stained agarose gel of the β-actin RT-PCR analysis (20 cycles). (Bottom) Autoradiograms of DNA blots of the $\lambda 5$ and *VpreB* RT-PCR amplification products (30 cycles). The cDNA from 70Z/3 cells was diluted 5-fold for the $\lambda 5$ and *VpreB* RT-PCR analyses.

The $\lambda 5$ Enhancer/Promoter Regions Contain Multiple EBF Binding Sites

The transcriptional activation of *VpreB* and $\lambda 5$ by EBF suggested that these markers may be direct or indirect genetic targets of this transcription factor. The transcriptional control of the $\lambda 5$ gene has been studied in transient transfection assays, which have identified a pre–B cell–specific enhancer/promoter activity between nucleotides -296 and $+65$ (Mårtensson and Melchers, 1994; Yang et al., 1995). Inspection of the nucleotide sequence of this transcriptional control region revealed multiple sequence elements that resemble the optimal EBF binding site (Travis et al., 1993) or a consensus E-box (Figure 3A). Based on the degeneracy of the potential EBF binding sites in the $\lambda 5$ gene, we examined whether oligonucleotides encompassing these sites could interfere with binding of EBF to its site in the *mb1* promoter (Figure 3B). Three oligonucleotides from the $\lambda 5$ gene that display sequence similarity with the EBF binding site competed efficiently with DNA binding by recombinant in vitro translated EBF. Sites 1 and 3 of the $\lambda 5$ enhancer/promoter region competed as efficiently as the EBF binding site in the *mb1* promoter, whereas site 2 competed with

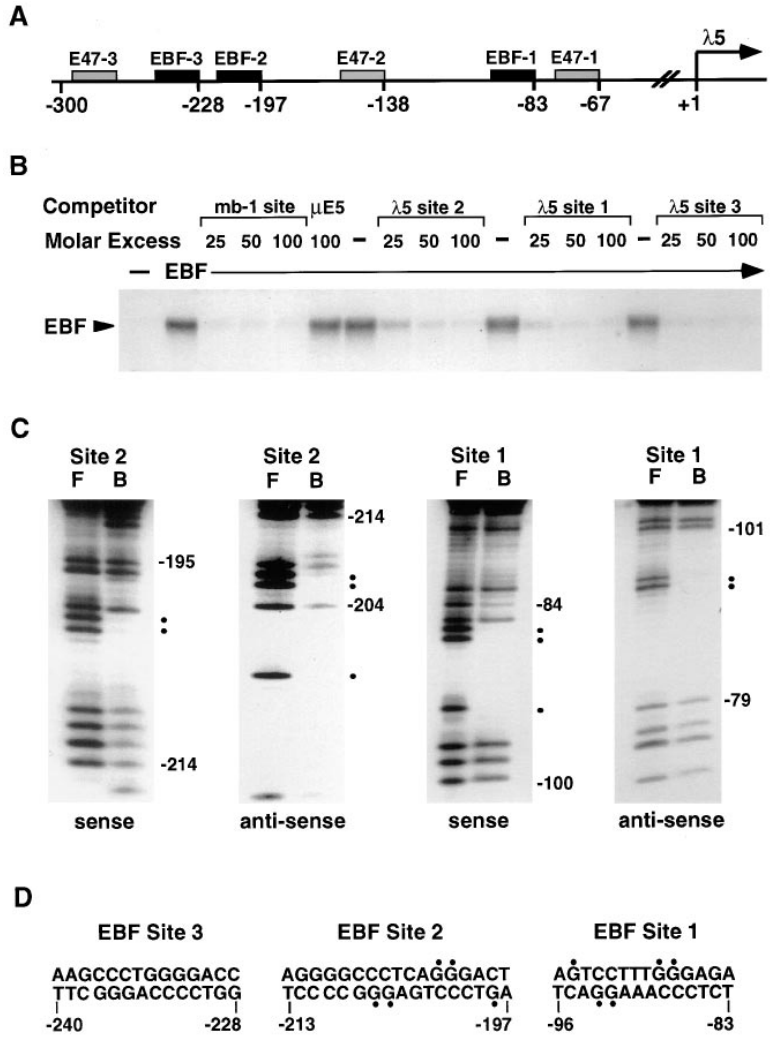

Figure 3. The λ*5* Enhancer/Promoter Region Contains Multiple EBF Sites

(A) Schematic diagrams of the λ*5* enhancer/promoter region with the positions of the EBF binding sites (solid boxes) and E-boxes (shaded boxes) indicated. Numbers below the line refer to the nucleotide positions, assigning the transcription start site as +1 (Yang et al., 1995).

(B) Binding of EBF to the λ*5* enhancer/promoter. Competition of the formation of the EBF–DNA complex by each of the three λ*5* EBF binding sites in an electophoretic mobility shift assay. Lane 1 contains 0.5 μl unprogrammed rabbit reticulocyte lysate. All other lanes contain 0.5 μl EBF-programmed lysate incubated with a labeled *mb1* oligonucleotide containing an EBF binding site. Fifty-, 100- or 200-fold molar excess of unlabeled duplex oligonucleotides was used for the competition of DNA binding by EBF as indicated. Unlabeled *mb1* and *μE5* oligonucleotides were used for the competition as positive and negative controls, respectively. The area of the gel containing the EBF–DNA complex is shown.

(C) Methylation interference assay of EBF binding to sites 1 and 2 of the λ*5* enhancer/promoter. Duplex oligonucleotides containing EBF binding sites 1 or 2 were methylated at guanosines and incubated with recombinant in vitro–translated EBF. Bound (B) and free (F) DNA were separated in an electrophoretic mobility shift assay and analyzed on a sequencing gel. Dots indicate methylated guanosines that interfere with EBF binding.

(D) Nucleotide sequences of the EBF binding sites in the λ*5* enhancer/promoter.

a 2-fold lower efficiency. No competition was observed with the unrelated *μE5* control oligonucleotide. We further defined EBF binding sites 1 and 2, which deviate from the consensus site, by methylation interference analysis. This analysis identified several specific nucleotides that are contacted by EBF (Figures 3C and 3D).

We confirmed binding of endogenous EBF to site 1 of the λ*5* enhancer/promoter region by electrophoretic mobility shift assays with nuclear extracts from 230–238 pre–B cells (Figure 4A). A single protein–DNA complex was detected that reacted with antiserum directed against EBF but not with antisera directed against the unrelated protein LEF-1.

To study the functional importance of the EBF binding sites in the λ*5* enhancer/promoter we transiently transfected 230–238 pre–B cells with reporter constructs containing flanking sequences (-299 to +131) from the λ*5* gene linked to the luciferase coding sequence (Figure 4B). Truncation of the λ*5* enhancer/promoter region to nucleotide −193, which removed two EBF sites and two E-boxes, decreased reporter gene expression to 10% of the level observed with the intact λ*5* enhancer/promoter region. Transfection of a deletion construct, in which an EBF site and two E-boxes were removed (Δ −141/−58), resulted in a similar decrease. We also examined directly the role of the EBF-binding sites in the λ*5* enhancer/promoter by introducing point mutations in site 1 and in both sites 1 and 2. These mutations reduced reporter gene expression to 30% and 7% of the wild type, respectively. Thus, the EBF binding sites are important for the function of the λ*5* enhancer/promoter region.

The promoter region of the *VpreB* gene also contains potential EBF and E47 binding sites (Kudo and Melchers, 1987). We confirmed by electrophoretic mobility shift assays that both recombinant EBF and E47 can bind to a *VpreB* promoter fragment, but the function of these sites was not examined further (data not shown).

Generation and Expression of an E47 Forced Homodimer

Although EBF induces the expression of λ*5* and *VpreB* in stably transfected Ba/F3 cells, the levels of expression are relatively low and can be detected only by RT-PCR analysis. In addition, the presence of multiple EBF and E47 binding sites in the λ*5* promoter and the similarity of the developmental arrest of B cell differentiation in EBF- and E47-deficient mice raised the question of

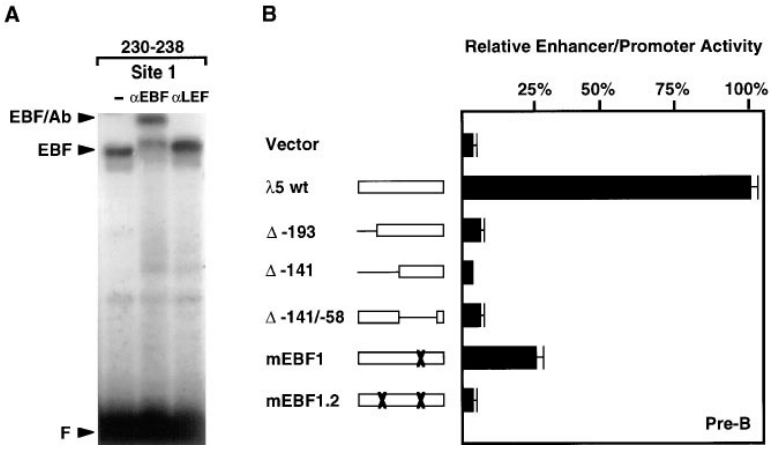

Figure 4. EBF Binding Sites Are Important for the Activity of the λ*5* Enhancer/Promoter in Pre–B Cells

(A) Electrophoretic mobility shift assay with nuclear extract from 230–238 pre–B cells. Five micrograms of nuclear extract was incubated with a labeled duplex oligonucleotide containing EBF site 1 of the λ*5* promoter, and the complexes were separated on a 6% polyacrylamide gel. The identity of the EBF–DNA complex was confirmed by reactivity with anti-EBF antiserum. F indicates migration of free probes.

(B) Functional importance of the EBF binding sites in the λ*5* enhancer/promoter region. (Right) The bar graph shows the results of transient transfections of 2 μg of wild-type or mutated λ*5* luciferase reporter genes into 230–238 pre–B cells. Luciferase activity was determined 2 days after transfection. Error bars indicate the maximal deviation from the mean value of four samples from two independent experiments. (Left) Lines represent deletions; crosses represent point mutations.

whether these transcription factors act in concert to regulate a set of genes that is essential for early B cell differentiation.

Functional analysis of E47 and the E47 homodimer, BCF-1, in non–B cells is complicated by the observation that the E47 homodimer is found only in B cells (Murre et al., 1991; Bain et al., 1993; Benezra, 1995; Shen and Kadesch, 1995). Although transient overexpression of E47 in fibroblasts allows BCF-1 formation and activation of endogenous genes (Choi et al., 1996), the relatively low frequency of gene transfer makes it difficult to quantitate the levels of expression of the induced genes on a per-cell basis. Moreover, the levels of expression of exogenous genes in transiently transfected cells typically exceed those of endogenous genes. Attempts to express E47 in stably transfected Ba/F3 cells did not generate any cell clones expressing high levels of BCF-1 (data not shown). This is consistent with previous reports that induced ectopic expression of E47 results in cell cycle arrest (Peverali et al., 1994). Therefore, we adopted the approach of generating a homodimeric fusion protein by linking two E47 molecules together in a head-to-tail configuration via a flexible glycine-rich linker (Figure 5A). This approach has been used successfully to generate functional MyoD–E47 covalent heterodimers in fibroblasts (Neuhold and Wold, 1993). Consistent with the formation of a covalent homodimer, in vitro–transcribed and –translated E47 forced dimer protein migrates with the predicted molecular mass of approximately 120 kDa in a denaturing gel (Figure 5B). To compare DNA binding by E47 and the E47 forced dimer, we examined binding of in vitro–translated proteins to a radiolabeled μ*E5* site in an electrophoretic mobility shift assay (Figure 5C). As anticipated, E47 bound DNA with low efficiency, whereas abundant DNA binding was detected with the E47 forced dimer. The complex formed between the E47 forced dimer and the μ*E5* DNA probe could also be supershifted by anti-E47 antibody but not by a control anti-T7 antibody. We confirmed the binding of the E47 forced dimer to the λ*5* enhancer/promoter region by showing that this DNA could compete with the formation of the E47–μ*E5* protein–DNA complex (Figure 5D). In a similar assay, the E47 forced dimer was also able to bind the *VpreB* promoter region (data not shown). In addition, we found that BCF-1 and the E47 forced dimer displayed similar DNA-binding specificity. Neither protein bound to the E-box binding site recognized by the bHLH-Zip protein USF (data not shown), and methylation interference assays showed that both E47 and the E47 forced dimer contact the same E-box DNA residues as those previously reported for E47 (Murre et al., 1989a). Thus, the covalent linkage of two E47 polypeptides generates a protein that binds DNA with high affinity and with similar specificity as native BCF-1.

EBF and E47 Synergistically Activate the λ*5* Enhancer/Promoter Region

To study potential cooperation between EBF and E47 in the activation of the λ*5* promoter, we transiently transfected λ*5* luciferase reporter gene constructs into Ba/F3 and HeLa cells together with expression plasmids encoding EBF or E47 (Figure 6A). Cotransfection of the λ*5* luciferase reporter construct with an EBF cDNA resulted in a 20-fold activation in Ba/F3 cells and a 3-fold activation in HeLa cells. In contrast, the E47 expression plasmid did not significantly increase the expression of the reporter gene in either cell type. Cotransfection of the reporter construct with both EBF and E47 expression plasmids markedly increased the activity of the λ*5* enhancer/promoter relative to that observed with EBF alone. The E47 forced dimer was functionally comparable to E47 in this transient transfection assay, and in combination with EBF, the forced dimer increased the activity of the λ*5* enhancer/promoter up to 240-fold. The transcriptional activation of the reporter gene by EBF and E47 was dependent on the presence of intact EBF binding sites, as shown by the decrease in activation of a reporter construct containing point mutations in all three EBF binding sites of the λ*5* enhancer/promoter. A control reporter containing the *fos* promoter was not affected by cotransfection with EBF and E47 forced dimers. In these experiments, we noted that the mutations in the three EBF-binding sites did not decrease λ*5* enhancer/promoter function to the same low levels as

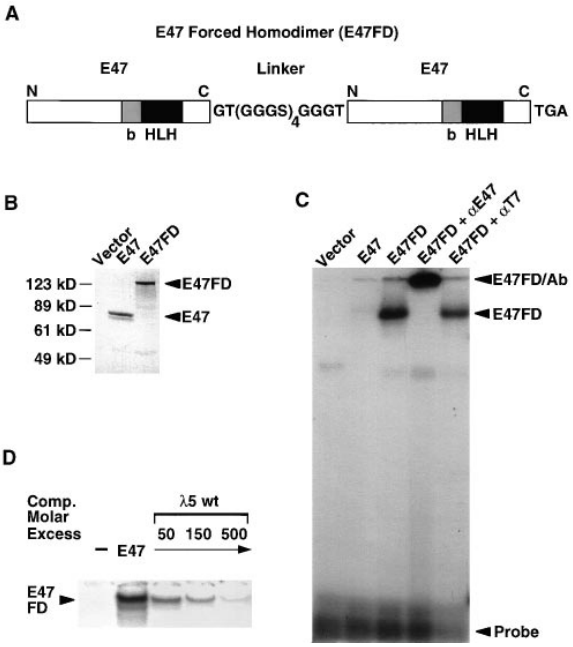

Figure 5. Covalent Linkage of Two E47 Molecules Results in an E47 Forced Dimer Polypeptide That Binds DNA Efficiently

(A) Schematic diagram of the fusion of two E47 molecules through a flexible linker. The bHLH DNA-binding domain is indicated.

(B) SDS-PAGE analysis of ^{35}S-labeled in vitro–translated E47 and E47 forced dimer. The E47 forced dimer migrates with an apparent molecular mass of 120 kDa.

(C) DNA binding by the E47 forced dimer. Electrophoretic mobility shift assay with unlabeled E47 and the E47 forced dimer, which were translated in parallel to the reactions used for the SDS-PAGE analysis. The polypeptides were incubated with a labeled *μE5* oligonucleotide in the absence or presence of anti-E47 or anti-T7 antibody.

(D) The E47 forced dimer binds the *λ5* enhancer/promoter region. Binding of the E47 forced dimer to a labeled *μE5* oligonucleotide in an electrophoretic mobility shift assay is reduced by addition of excess of unlabeled *λ5* DNA fragment (nucleotides −299 to +131). The region of the gel containing the E47–DNA complex is shown.

observed in the absence of EBF protein. This discrepancy may be due to the presence of low-affinity EBF sites in the *λ5* transcriptional control region that can be recognized by EBF in transfected cells that express the protein at high levels. Taken together, these data indicate that EBF and E47 collaborate in the activation of the *λ5* enhancer/promoter.

To examine the effect of the EBF site point mutations in the *λ5* enhancer/promoter and to determine whether binding of EBF to the three sites involves cooperativity, we examined the ability of various *λ5* enhancer/promoter fragments to compete for binding of EBF to the *mb1* duplex oligonucleotide (Figure 6B). Competition with the wild-type enhancer/promoter fragment was only 3-fold more efficient than with a fragment containing point mutations in two EBF-binding sites, suggesting that EBF binds to the sites independently of cooperative interactions. As a control, no competition was observed with the enhancer/promoter carrying mutations in all three EBF sites.

We also investigated the contribution of E-boxes to the observed synergy between EBF and E47. Toward this end, we mutated E47 site 1, alone or in combination with EBF site 1, and transiently transfected the mutated *λ5* reporter gene construct into 230–238 pre–B cells (Figure 6C). Mutation of E47 site 1 decreased the activity of the *λ5* promoter 2-fold, whereas mutation of EBF site 1 decreased promoter activity 4-fold. Mutation of both factor-binding sites resulted in a 10-fold decrease in the activity of the *λ5* promoter. These data suggest that both EBF and E47 binding sites contribute to the activity of the *λ5* promoter, although we did not detect any significant synergy between the two factor-binding sites examined. Consistent with this, we failed to observe cooperative DNA binding by EBF and the E47 forced dimer to a *λ5* DNA fragment containing two E-boxes and EBF site 1 (data not shown). Thus, the synergy between EBF and E47 may depend on the multiplicity of their binding sites in the *λ5* enhancer/promoter.

With the aim of examining the potential of EBF and E47–E47 homodimers to cooperatively induce the expression of the endogenous *λ5* gene, we transiently transfected Ba/F3 cells with EBF and E47 expression plasmids. Analysis of the transfected cells using an RT-PCR assay indicated that the endogenous *λ5* gene was activated by EBF, but not by either E47 or the E47 forced dimer (Figure 6D). Cotransfection of EBF and either E47 or the E47 forced dimer did, however, induce higher levels of expression from the *λ5* gene, suggesting that EBF and E47 can cooperate to induce expression of the endogenous *λ5* gene, as was observed in the stable transfections of Ba/F3 cells. Induction of *λ5* expression could also be observed upon transient transfection of EBF and the E47 forced dimer expression plasmids into NIH 3T3 fibroblasts, suggesting that activation of the endogenous *λ5* gene is not specific for Ba/F3 cells.

Synergistic Transcriptional Activation of the Endogenous *λ5* and *VpreB* Genes by EBF and E47

To study the effects of expressing physiological levels of E47 homodimers, alone or in combination with EBF, we generated stable transfectants in Ba/F3 cells and examined protein expression by electrophoretic mobility shift assays of nuclear extracts (Figures 7A and 7B). We obtained five clones that expressed the E47 forced dimer alone at levels comparable to the level of endogenous BCF-1 in the pre–B cell line 70Z/3 (Figure 7A). However, none of the clones expressed *λ5* or *VpreB* at levels that could be detected by 30 cycles of RT-PCR (data not shown). Moreover, neither *Pax5*, *IL7R*, *Rag1*, nor *TdT* transcripts were detected in this assay. Previous data suggested that Iμ is a target for E47 (Schlissel et al., 1991; Sloan et al., 1996); however, because of the presence of *I*μ transcript in the parental Ba/F3 cell line, we were unable clearly to show consistent up-regulation of *I*μ by RT-PCR in the clones that expressed the E47 forced dimers.

We further examined the possibility that EBF and E47 collaborate in the transcriptional activation of B cell–specific genes by generating stably transfected Ba/F3 cell lines that express both EBF and the E47 forced dimer. In cotransfections of the E47 forced dimer gene

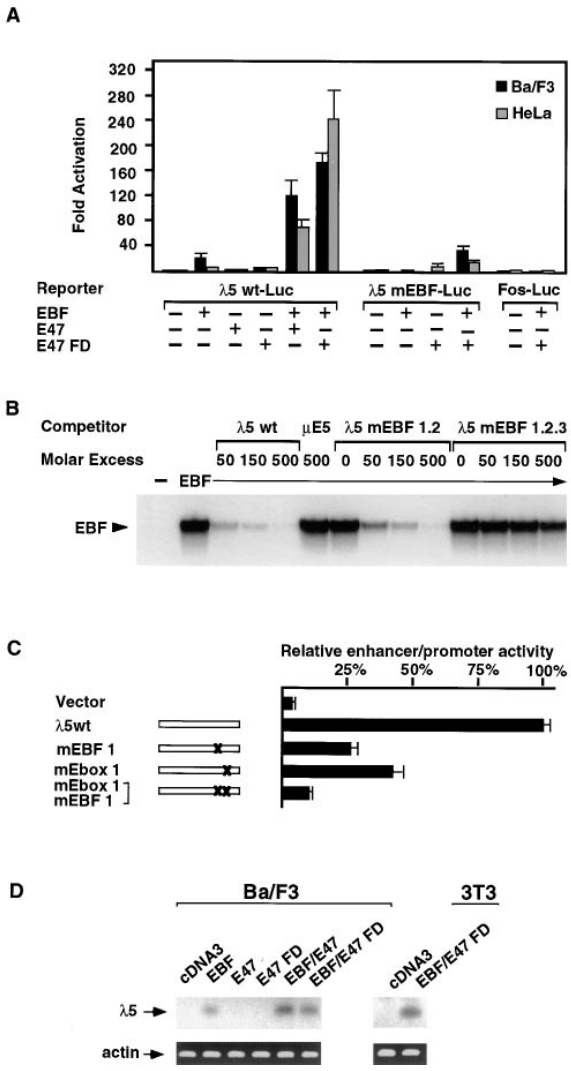

Figure 6. Synergistic Activation of the λ*5* Promoter in Ba/F3 and HeLa Cells by EBF and E47

(A) Ba/F3 and HeLa cells were transiently transfected with 0.5 μg of the λ*5* luciferase reporter gene construct, containing nucleotides −299 to +131 of the λ*5* gene, together with 250 ng of EBF, 250 ng of E47, or 250 ng of E47 forced dimer (E47FD) cDNA expression plasmids alone or in pairwise combination as indicated. The levels of luciferase activity were normalized for the expression of a cytomegalovirus β-galactosidase reporter plasmid, and the data are the average of four transfections. Fold induction was quantitated relative to the level of luciferase activity obtained with an expression vector lacking a cDNA insert. The λ*5* mEBF luciferase reporter gene construct contains point mutations in all three EBF binding sites (see Experimental Procedures).

(B) Electrophoretic mobility shift assay of EBF binding to the λ*5* enhancer/promoter. In vitro–translated EBF was incubated, in the absence or presence of competitor DNA, with a labeled oligonucleotide containing the *mb1* EBF-binding site. For the competition, wild-type λ*5* DNA (nucleotides -299 to +131) and mutant λ*5* DNA containing point mutations in EBF site 1 or 2 or in all three EBF sites was used. The area of the gel containing the EBF–DNA complex is shown.

(C) Contribution of an E-box to the regulation of the λ*5* promoter. Wild-type and mutated λ*5* luciferase reporter gene constructs (2 mg) were transiently transfected into 230–238 pre–B cells. The mutations in the EBF site 1 and E-box 1 of λ*5* enhancer/promoter (-299 to +131) are represented by crossed bars. The luciferase activity represents the average values obtained from four samples and two independent experiments.

(D) Induction of endogenous λ*5* gene expression by transient ectopic expression of EBF and E47. Ba/F3 cells or NIH 3T3 cells were transiently transfected with 15 μg of the indicated expression plasmids. RNA was isolated after 48 hr and analyzed by RT-PCR analysis for expression of actin (20 cycles) and λ*5* (30 cycles for Ba/F3 cells and 35 cycles for NIH 3T3 cells).

construct and a puromycin-resistance marker into one of the EBF-expressing Ba/F3 cell lines, only 2 of 45 clones were obtained that expressed both transcription factors, as determined in electrophoretic mobility shift assays (Figure 7B). In these experiments, we noted that most E47 forced dimer–expressing clones had lost expression of EBF, despite being kept under double selection, implying a selection against cells expressing both proteins. In the two Ba/F3 clones that expressed both EBF and the E47 forced dimer, RT-PCR showed that λ*5* and *VpreB* transcripts were relatively more abundant than in two EBF-positive clones that expressed comparable amounts of EBF (data not shown). To quantitate the levels of target gene expression, we examined these B cell–specific transcripts by S1 nuclease protection assays of total RNA (Figure 7C). In Ba/F3 cell clones expressing either EBF or the E47 forced dimer alone, no λ*5* or *VpreB* transcripts were found, whereas in the two cell clones expressing both transcription factors, λ*5* transcripts were readily detected. The levels of expression were calculated at 84% and 17% of the levels detected in the pre–B cell line 230–238, when normalized to the levels of the β-*actin* control transcript. The clones expressing both EBF and the E47 forced dimer, were also found to contain *VpreB* RNA at 19% and 3% of the level detected in 230–238 cells. Thus, EBF and the E47 forced dimer synergize in activating expression of the endogenous λ*5* and *VpreB* loci from a transcriptionally silent state in Ba/F3 cells.

Discussion

The transcription factors EBF and E47 have been shown to regulate early stages of B cell differentiation (Bain et al., 1994; Zhuang et al., 1994; Lin and Grosschedl, 1995). The pronounced similarity of the phenotypes of EBF- and E47-deficient mice raised the possibility that these transcription factors regulate a similar set of genes. However, the complete block of B cell differentiation in both mutant strains of mice has complicated the identification of important target genes. Here we show that the transcription control regions of both the λ*5* and *VpreB* genes contain multiple EBF- and E47-binding sites, indicating that these genes are direct targets of EBF and E47. We also find that the λ*5* enhancer/promoter region can be activated strongly by EBF and E47 in transiently

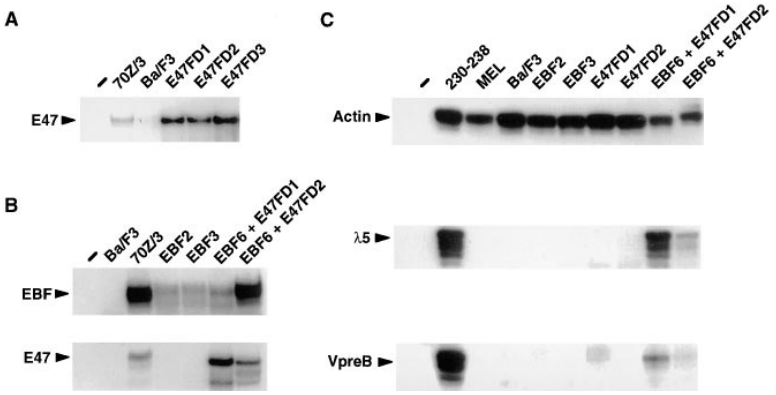

Figure 7. Stable Coexpression of E47 Forced Dimer and EBF Induces High Levels of λ*5* and *VpreB* in Ba/F3 Cells

(A) Electrophoretic mobility shift assay to detect E47 DNA-binding activity in Ba/F3 cells transfected with E47 forced dimer cDNA. Five micrograms of nuclear extract was incubated with a labeled μ*E5* oligonucleotide probe and separated on a 6% polyacrylamide gel. The region of the gel containing the E47 forced dimer (E47FD)–DNA complex is shown.

(B) Electrophoretic mobility shift assay to detect E47 and EBF DNA-binding activity in Ba/F3 cells transfected with both EBF and E47 forced dimer cDNAs. Five micrograms of nuclear extracts from 70Z/3 pre–B cells and untransfected and transfected BaF/3 cell clones was incubated with a labeled μ*E5* oligonucleotide (top) or an *mb1* oligonucleotide containing an EBF-binding site.

(C) EBF and E47 collaborate to promote high levels of transcription from the λ*5* and *VpreB* genes in stably transfected Ba/F3 cells. Autoradiograms show the results of S1 nuclease protection assays of total RNA from 230–238 pre–B cell, the parental Ba/F3 cells, or Ba/F3 clones that have been stably transfected with EBF or E47 forced dimer alone or with both EBF and E47 forced dimer. Specifically protected fragments are detected in the 230–238 pre–B cells and in the Ba/F3 clones expressing both EBF and E47 forced dimer. (Top) The results of the S1 nuclease protection analysis with an end-labeled β-actin probe and 5 μg of total RNA. (Bottom) The S1 nuclease protection analysis with end-labeled λ*5* and *VpreB* probes and 25 μg and 10 μg of total RNA, respectively.

transfected HeLa cells in a site-dependent manner. Finally, stable expression of EBF and E47 homodimers in immature hematopoietic Ba/F3 cells induces transcription of the genes encoding the Ig surrogate light chains λ*5* and *VpreB*. Notably, EBF and E47 act in concert to induce the expression of the endogenous λ*5* and *VpreB* genes from a transcriptionally silent state to levels approaching those found in pre–B cell lines. Induction of endogenous λ*5* gene expression by EBF and E47 can also be observed in transiently transfected Ba/F3 and NIH 3T3 fibroblastic cells.

Cell type–specific expression of genes is governed by transcription factors that are restricted in their cell type distribution. In yeast, a simple paradigm for cellular differentiation, a single transcription factor is sufficient to induce a specific cellular phenotype. Ectopic expression of the α*1* gene in cells of the α mating type induces an entire set of a specific genes (reviewed by Herskowitz, 1989). In higher eukaryotes, each of a set of myogenic bHLH proteins, MyoD, myogenin, Myf-5, and MRF-4, can induce morphological changes and transcriptional activation of muscle-specific genes when introduced into non–muscle cell types (reviewed by Olson, 1990; Weintraub, 1991; Emerson, 1993). The efficiency of MyoD-induced myogenesis typically is low but can be significantly enhanced by coexpression of any of these bHLH proteins with the transcription factor MEF2 (Molkentin et al., 1995). A similar cooperation between two cell type-specific transcription factors C/EBPα and PPARγ2 is required for the efficient induction of adipogenesis in fibroblastic cell lines (Tontonoz et al., 1994). The synergy of EBF and E47 homodimers in activating the pro B cell markers λ*5* and *VpreB* resembles the cooperation of cell type-transcription factors in myogenesis and adipogenesis. In contrast to those differentiation systems, the collaboration of EBF and E47 results in the induction of only a small subset of lineage specific markers rather than an entire differentiation program. This difference may reflect the multistep process of B cell differentiation (reviewed by Alt et al., 1987; Melchers et al., 1993) or the inability of EBF and E47 homodimers to function as lineage commitment factors.

Various mechanisms have been shown to contribute to synergy between transcription factors. First, cooperative DNA binding by two transcription factors can increase both the affinity of DNA binding and the diversity of the transcriptional response. For example, the yeast α2 repressor protein, which alone binds DNA inefficiently, can be recruited to its site by cooperative binding with MCM protein, which by itself functions as an activator of transcription (Keleher et al., 1988). Second, collaboration between distinct transcription factors may also involve protein–protein interactions independent of changes in DNA binding. The transcriptional activation potential of MyoD–E12 heterodimers can be greatly augmented by their association with the DNA-binding domain of MEF2. Although EBF and E47 contain dimerization motifs that share sequence similarities and both proteins display a strong functional synergy in activating pre–B cell–specific genes, we have been unable to coimmunoprecipitate in vitro–translated EBF and E47 (data not shown). Moreover, we find that coincubation of EBF and E47 homodimers with a probe containing both binding sites yields predominantly single factor–DNA complexes. Thus, the synergy between EBF and E47 in the regulation of the λ*5* gene may be independent of direct protein–protein interactions. Instead, the multiplicity of binding sites for EBF and E47 may account for the magnitude of the collaborative activation of gene expression. It is also possible that multiple binding sites for regulatory factors such as EBF and BCF-1 may facilitate the activation of target genes in the context of nuclear chromatin.

EBF and E47 homodimers appear to differ in their potential to activate transcription of the λ*5* and *VpreB* genes. EBF, but not the E47 forced dimer, is able to induce low levels of expression of the endogenous λ*5* and *VpreB* genes in Ba/F3 cells. This observation raises the possiblities that EBF may be the primary determinant in the activation of the silent λ*5* and *VpreB* loci and that

E47 collaborates with EBF to significantly increase the level of transcription. E47 has been shown previously to act as a potent transcriptional activator (Quong et al., 1993), and consistent with this, the E47 forced dimer is capable of activating the λ*5* promoter in transient transfection assays (Figure 6A). In addition, the relative levels of transcription of the λ*5* and *VpreB* genes in the two Ba/F3 cell clones that express both EBF and the E47 forced dimer correlates with the abundance of the E47 forced dimer and not with that of EBF. Thus, EBF may be capable of acting on regulatory regions in the context of transcriptionally inactive chromatin, whereas E47 may provide strong transcriptional activation functions.

In addition to EBF and E47, other B cell–specific transcription factors may contribute to the regulation of the λ*5* and *VpreB* genes. For example, both the λ*5* and *VpreB*1 enhancers contain binding sites for Pax5 (BSAP and EBB-1) (Okabe et al., 1992b; Yang et al., 1995) and the absence of *Pax5* transcripts in Ba/F3 cells could account for the lower levels of *VpreB* and λ*5* gene expression as compared to pre–B cells. It is also important to note that the expression patterns of EBF, *VpreB*, and λ*5* are overlapping but not identical, either in tissue distribution (Hagman et al., 1993; Wang and Reed, 1993) or during B cell differentiation (Kudo and Melchers, 1987; Kudo et al., 1987; Hagman et al., 1991, 1993; Okabe et al., 1992a). The more restricted distribution of λ*5* and *VpreB* may be explained by the presence of repressor elements in the control regions of these genes (Mårtensson and Melchers, 1994; Okabe et al., 1992a), which may be important in both spatial and temporal regulation.

The E47 protein has been previously shown to regulate the progression of the cell cycle (Peverali et al., 1994). Experiments in which the levels of E47 protein were increased either with an inducible expression system in stably transfected NIH 3T3 cells or with microinjection of antibodies directed against endogenous Id indicated that E47 has a cell growth–suppressive activity (Peverali et al., 1994). The HLH protein Id forms a heterodimeric complex with E47 that is unable to bind DNA (Benezra et al., 1990) and overexpression of Id1 in transgenic mice results in a block of B cell differentiation with a markedly reduced level of λ*5* transcripts (Sun, 1994). In our stable transfection experiments with the E47 forced dimer, we have not observed any significant change in the proliferation of cells. This may be due to our choice of an immature hematopoietic cell line instead of fibroblast cells for our transfections or, alternatively, may suggest that the cell cycle block is a result of a heterodimeric complex of E47 with another protein.

Previous experiments in which E47 cDNA had been transiently or stably transfected into fibroblasts or pre–T cells identified the *Rag1*, *I*μ, and *TdT* genes as targets for E47 (Schlissel et al., 1991; Choi et al., 1996). We did not detect significant levels of *TdT* or *Rag1* transcripts in Ba/F3 cells stably transfected with either the E47 forced dimer alone or with the E47 forced dimer in combination with EBF, nor was *I*μ up-regulated consistently. The ectopic activation of the *Rag1*, *TdT*, and *I*μ genes, which are lymphoid specific rather than B cell specific, may be mediated by E47 in complex with another protein.

Our data strongly suggest that one function of the transcription factors EBF and E47 is to activate a subset of early B cell–specific genes. Although our data identify the λ*5* and *VpreB* genes as genetic targets for EBF and E47, the targeted inactivation of the λ*5* gene results in a phenotype distinct from the early block of B cell differentiation in EBF- and E47-deficient mice (Kitamura et al., 1992; Bain et al., 1994; Zhuang et al., 1994). A targeted inactivation of λ*5* and both functionally equivalent murine *VpreB* genes (Kudo and Melchers, 1987) may be needed to address the question of whether the combined absence of these proteins can account for the early block of B cell differentiation in the EBF- or E47- deficient mice. It is also possible that other genes are regulated by EBF and E47. We have considered *mb1* to be a candidate target gene for EBF because of the presence of a functionally important site in the promoter that binds EBF (Hagman et al., 1991). Furthermore, EBF and mb-1 share a similar expression pattern in the B cell lineage (Hagman et al., 1991, 1993; Travis et al., 1991). However, the *mb1* gene is expressed in Ba/F3 cells in the absence of EBF and BCF-1 and of Pax5, which binds the *mb1* promoter in cooperation with an Ets family protein (Fitzsimmons et al., 1996). Thus, only a subset of cell type specific genes that contain binding sites for a particular transcription factor may represent in vivo genetic targets. Further experiments will be required to define any additional functions of EBF and E47 during B-cell development and maturation.

Experimental Procedures

Cell Lines and Flow Cytometry

Lymphoid cells (70Z/3 and 230–238) were maintained in RPMI (GIBCO) supplemented with 50 μM β-mercaptoethanol, 1 mg/ml penicillin–streptomycin, and 10% fetal calf serum. For the maintenance of Ba/F3 cells the culture medium was supplemented with 10% conditioned medium from WEHI 3 cells as a source of IL-3. HeLa cells were grown in DMEM (GIBCO) supplemented with 1 mg/ml penicillin–streptomycin and 5% fetal calf serum.

For flow cytometry, 10^6 cells were washed with phosphate-buffered saline containing 3% bovine serum albumin and incubated 30 min with phycoerythrin-conjugated rat anti-mouse B220 antibody (Caltag). The cells were washed twice in phosphate-buffered saline/bovine serum albumin, and the samples were analyzed in a FACScalibur flow cytometer using CellQuest software (Becton Dickinson).

Gene Constructs and Site-Directed Mutagenesis

The EBF cDNA used in all the experiments was modified at the C-terminus with a T7 tag sequence by PCR, using the primers: EBF-PVU2 5′-CTTCCTCAATGGCTCAGCTGC-3′ and T7CTag/EBF 5′-GATCTAGACTAGCCCATCTGCTGGCCGCCGGTCATGCTCATGGG AGGGACAATCATGC. Stable cell lines were made with EBF-T7 in pcDNA3 (Invitrogen). The EBF-T7 cDNA3 construct contains 45 nucleotides of the 5′ untranslated region. The E47 forced dimer construct was generated by joining two copies of a syrian hamster E47 cDNA, *E47mutC*, and *E47mutN*, with a linker encoding multiple glycines and serines. *E47mutN* incorporates an NdeI site at the N-terminal ATG of E47. *E47mutC* contains an XhoI site replacing the C-terminal stop codon. The reconstruction sites were introduced by site-directed mutagenesis: *E47mutC* 5′CCGGGCACCTCGAGCT GCCACATGG and *E47mutN* 5′-GCCCTGGCCATATGATGAACC. *E47mutC* was digested with XhoI and ligated to the peptide linker which contains an N-terminal XhoI site and a C-terminal NdeI site (peptide linker 5′-TCGAGGGGACAGGGGGGGGGAGCGGGGGGGG GAGCGGGGGGGGGAGCGGGGGGGGGAGCGGGGGGGGGACACA) to produce the construct *E47mutC*/linker and *E47mutN*, which were

then digested with NdeI and XbaI. The appropriate digestion fragments were ligated together to produce the E47 forced dimer construct in pcDNA3 (Invitrogen).

The λ*5* enhancer was cloned by PCR amplification from genomic DNA using primers amplifying a 430 bp fragment containing sequences from nucleotide −299 to the translation start site at nucleotide +131. The primers (sense: 5′-GGGGTACCAGAGACTCTTGTTC CATGG; antisense: 5′-CCGCTCGAGTCTAGCCTCACTTGCAG) included restriction sites to facilitate the cloning of the enhancer into the KpnI and XhoI sites of the luciferase reporter vector, pGL3 basic (Promega). Point mutations were introduced by combinations of PCR and restriction enzyme digestions to generate the mutant enhancers. Primers for PCR mutagenesis were used in combination with the λ*5* primers described above: mEBF site 1 antisense 5′-CCA TGGTCACCATCTGTGGAGTTCTCTATTAAATTACTACCCGGTTGTG; mEBF site 2 sense: 5′-CCAGGGGCCCTAATATACTGGATATCAG TCAGGC; mEBF site 3 antisense 5′-TATTAGGGCCCCTGGGTCTGT GGAGCAGGTAGCTACTGCTTAGAGGGGCC; and mE-box 1 antisense 5′-CCATGGTCACCTTCCGTGGAGTTCTCT.

Stable Cell Transfections

Ba/F3 cells were transfected with the expression vector pcDNA3 containing EBF-T7 or E47 forced dimer cDNA under the control of the cytomegalovirus promoter. First, 10^7 cells were electroporated (960 μF, 250V) with 2 μg of plasmid in 0.5 ml of medium. After 24 hr, the cells were diluted in 150 ml of medium containing 0.75 mg/ml active neomycin (Geneticin, GIBCO). The cells were plated into 24-well plates and incubated until colonies were visible (10–14 days). Cells from wells containing single colonies were expanded, and nuclear extracts were prepared as described by Schreiber et al. (1989). Double-transfected Ba/F3 cell lines were generated by sequential transfections of 2 μg of EBF cDNA plasmid containing a linked neomycin resistance gene, *neoR*, and 10 μg of E47 forced dimer cDNA construct together with 1 μg of the puromycin resistance gene. Neomycin- and puromycin-resistant cell clones were screened for the presence of both the E47 forced dimer and EBF activity in electrophoretic mobility shift assays (see below).

Transient Transfections and Luciferase Assays

2×10^6 230–238 pre–B cells, Ba/F3 cells, or HeLa cells were washed with TS (140 mM NaCl, 5 mM KCl, 25 mM Tris [pH 7.4], 0.4 mM sodium phosphate, 50 mM $MgCl_2$, 90 mM $CaCl_2$) and transfected with 0.5–2 μg of reporter gene construct as indicated together with 0.5 μg RSV-β-galactosidase control plasmid in 0.5 ml TS with 0.5 mg/ml DEAE dextran (Pharmacia) and 0.2 mM chloroquine as described (Grosschedl and Baltimore, 1985). The transfected cells were incubated for 36 hr.

Preparation of protein extracts and luciferase assays were performed with a luciferase assay kit (Promega) using 10% of the protein extract. β-Galactosidase assays were performed with 10% of the same protein extract in 40 μl of reaction buffer (150 mM Tris [pH 7.5], 10 mM $MgCl_2$, 25 mM NaCl, 10 mM β-mercaptoethanol, 1 mg/ml BSA, 0.15 mM 4-methylumbelliferyl β-galactosidase) for 30 min at room temperature. The reactions were stopped by the addition of 1 ml of 0.25 M glycine (pH 10.65), and the conversion of substrate was measured in a Hoefer DNA fluorometer TKO 100.

Transient transfections to study the induction of the endogenous λ*5* gene were done by electroporation (see above) of 5×10^6 cells with 15 μg of expression plasmid. The cells were harvested after 48 hr and RT-PCR analysis was performed as described below.

Protein Extracts and Electrophoretic Mobility Shift Assay

Nuclear extracts were prepared according to Schreiber et al. (1989). Protein concentrations were estimated by measuring the absorbance at 280 nm. DNA probes were labeled with γ[^{32}P]ATP by incubation with T4 polynucleotide kinase, annealed, and then purified on a 5% polyacrylamide TBE gel. Five micrograms nuclear extract or 1 μl of in vitro–transcribed/translated protein (Promega TNT) was incubated with labeled probe (20,000 cpm, 3 fmol) for 30 min at room temperature in binding buffer (10 mM HEPES [pH 7.9], 70 mM KCl, 1 mM DTT, 1 mM EDTA, 2.5 mM $MgCl_2$, 0.05% NP40) with 0.75 μg poly(dI-dC) (Pharmacia). DNA competitors and antibodies were added 10 min before the addition of the DNA probe. The samples were separated on a 6% acrylamide TBE gel, which was dried and subjected to autoradiography. Data were quantitated on a PhosphorImager, using ImageQuant software (Molecular Dynamics).

Oligonucleotides used for electrophoretic mobility shift assays were as follows: *Oct* sense 5′-GATCTGCTTCTTAATAATTTGCATAC CCTCACTG; *Oct* antisense 5′-GATCCAGTGAGGGTATGCAAATTAT TAAGAAGCA; μ*E5* sense 5′-GGCCAGAACACCTGCAGACG; μ*E5* antisense 5′-CGTCTGCAGGTGTTCTGGCC; *mb1* EBF sense 5′-GAGA GAGACTCAAGGGAATTGTGG; *mb1* EBF antisense 5′-CCACAAT TCCCTTGAGTCTCTCTC; λ*5* EBF 1 sense 5′-TCGACAACCGGGTA GTCCTTTGGGAGA-GAACTCCACAGATGG; λ*5* EBF 1 antisense 5′-TCGACCATCTGTGGAGTTCTCTCCC-AAAGGACTACCCGGTTG; λ*5* EBF 2 sense 5′-TCGACCCAGGGGCCCTCAGGGACTG-GATAT CAG; λ*5* EBF 2 antisense 5′-TCGACTGATATCCAGTCCCTGAGGGC CCCTGGG; λ*5* EBF 3 sense 5′-CCCCTCTAAGCCCTGGGGACCTGC TCCACAGAC; and λ*5* EBF 3 antisense 5′-GTCTGTGGAGCAGGTCC CCAGGGCTTAGAGGGG.

Reverse Transcriptase and Polymerase Chain Reactions

RNA was prepared from cells using an RNA extraction kit (Biotex Inc.) cDNA was generated by annealing 1 μg of total RNA and 1 pmol of random hexamers (Pharmacia) in 10 μl of DEPC-treated water. Reverse transcriptase reactions were performed with 2 units of MMLV-RT (Boehringer) in the manufacturer's buffer supplemented with 1 mM dNTP and 0.05 units/ml RNAsin (Promega) in a total volume of 20 μl at 37°C for 1 hr. One twentieth of the RT reaction was used for the PCR assays.

PCR reactions were performed with 1 unit of Taq-polymerase (Boehringer Mannheim) in the manufacturer's buffer supplemented with 0.2 mM dNTP in a total volume of 10 ml. The condition of each PCR cycle was (unless indicated otherwise) 94°C, 30 sec; 55°C, 1 min; and 72°C, 45 sec. Primers were added to a final concentration of 1 mM. Twenty-two cycles were used for *actin* RT-PCR and 30 cycles for all other primer sets. Primers used were as follows: *actin* sense 5′-GTTTGAGACCTTCAACACC; *actin* antisense 5′-GTGGCCA TCTCCTGCTCGAAGTC; *B29* sense 5′-GGTGAGCCGGTACCAG CAATG; *B29* antisense 5′-AGTTCCGTGCCAC AGCTGTCG; *VpreB* sense 5′-CGTCTGTCCTGCTCATGCT; *VpreB* antisense 5′-ACGGC ACAGTAATACACAGCC; λ*5* sense 5′-TGTGAAGTTCTCCTCCTG CTG; λ*5* antisense 5′-ACCACCAAAGTACCTGGGTAG; *mb1* sense 5′-GCCAGGGGGTCTAGAAGC; *mb1* antisense 5′-TCACTTGGCAC CCAGTACAA; *Iμ* sense 5′-ACCTGGGAATGTATGGTTG TGGCTT; *Iμ* antisense 5′-ATGCAGATCTCTGTT-TTTGCCTCC; *Rag1* sense 5′-TGCAGACA TTCTAGCACTCTGG; *Rag1* antisense 5′-ACATCT GCCTTCACGTCGAT; *TdT* sense 5′-GAAGATGGGAACAACTCG AAGAG; *TdT* antisense 5′-TGGCAGAGATTTCA GTACAGAGG; *Pax5* sense 5′-CTACAGGCTCCG-TGACGCAG; *Pax5* antisense 5′-GTC TCGGCCTGTGAAATAGG; *E47* sense 5′-GACGCCGAAGAGGACAA GAA; and *E47* antisense 5′-CAGGATCACCGTCACCGCCT.

In Vitro Transcription and Translation

Recombinant proteins were generated by coupled in vitro transcription/translation using a reticulocyte lysate kit (Promega, TNT). One microliter of a 25 μl reaction mix was used for electrophoretic mobility shift assays. Labeled proteins were translated in the presence of 20 mCi ^{35}S methionine and 1 μl of the reaction was separated on a 10% SDS PAGE gel. The gel was dried and subjected to autoradiography.

Methylation Interference and S1 Nuclease Protection Assays

Methylation of 5′-^{32}P–labeled oligonucleotides was performed as described (Hagman et al., 1991). Methylated DNA was incubated with in vitro–transcribed/translated EBF, and the bands representing bound and free DNA were excised, extracted in Tris-EDTA, ethanol precipitated, boiled in 1 M piperidine for 25 min, lyophilized three times, resuspended in sequence loading buffer, and separated on a 10% sequencing gel.

S1 nuclease protection assays were performed essentially as described in Grosschedl and Baltimore (1985). 5′-^{32}P–labeled DNA probes for S1 mapping were generated by primer extension of radiolabeled oligonucleotides on a plasmid template. Oligonucleotides used were actin 5′GGCCATCTCCTGCTCGAAGTC; λ*5* 5′CCTACTCT

GAGCTTCATTGACC; and *VpreB* 5′GGACAGACGTCCAGGCCATG. The extension product was separated on a 6% denaturating acrylamide gel, purified, and hybridized with total RNA at 45°C. The hybrids were digested with 30 units of S1 nuclease at 37°C for 1 hr and separated on a 6% denaturing polyacrylamide-urea gel. Data were quantitated on a PhosphorImager, using ImageQuant software (Molecular Dynamics).

Acknowledgments

Correspondence should be addressed to R. G. (e-mail: rgross@itsa.ucsf.edu). We thank Dr. Michael German (University of California, San Francisco) for providing the hamster E47 cDNA and Ross Okamura for assistance with the flow cytometry analysis. We are grateful to Drs. Tris Parslow, George Farmer, and Tannishtha Reya for critical reading of the manuscript and to Jacqueline Bruszewski-Walters for expert preparation of the manuscript. M. S. was supported by a postdoctoral fellowship award from the Swedish Medical Research Council. This work was supported by funds from the Howard Hughes Medical Institute.

Received April 7, 1997.

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